

To United States Patent and Trademark Office:

Re: U.S. Serial No. 10/522,110

Completion of PCT/CN2003/000609

Based on Chinese Patent Application No. 02125917.8

### DECLARATION OF INVENTOR

I, the undersigned, Xu Qishou, declare and say:

1. I am a citizen of the People's Republic of China and I reside in Beijing, P.R.C.
2. I am a professor at the Institute of Radiation Medicine, Academy of Military Science, People's Liberation Army, Beijing, China, and I have been doing researches relating to the art of the present invention for more than 30 years.
3. I am one of the co-inventors of Chinese Patent Application No. 02125917.8, filed on August 2, 2002, entitled "A Riboflavin Derivative and Its Manufacture and Uses", which is the priority application of the present U.S. patent application Serial No. 10/522,110.
4. I am familiar with the references cited by the examiner in the captioned application.
5. I am aware of and helped structure various tests conducted to assess riboflavin derivatives, including three sets of tests conducted to assess the effects of: (1) *in vitro* hydrolysis of riboflavin derivatives; (2) conducting an oral administration of riboflavin derivatives; and (3) conducting an intramuscular injection administration of riboflavin derivatives.
6. The tests are described in detail in Annex I and I will refer to the results obtained through the tests.
7. In my opinion, the presently claimed invention, as evidenced by the results shown in Annex I, offers clearly unexpected results of using 5'-lauric acid as opposed to those known in the art, especially di-, tri-, and tetra-ester of riboflavin.
8. In my opinion, the selection of the specific esterification degree (monoester), esterification site (5'-), and ester-forming carboxylic acid (lauric acid), as claimed and shown in Annex I, offers an unexpected technical effect of providing a superior long-acting property.
9. I declare that all statements made herein of my own knowledge are true

and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Qishou, Xu



Professor of Institute of Radiation  
Medicine, Academy of Military  
Medical Sciences, People's  
Liberation Army

Date: January 11, 2010

**ANNEX I OF DECLARATION OF QISHOU XU FOR U.S. PATENT  
APPLICATION NO. 10/522,110**

The following riboflavin derivatives are used in the tests:

1. *Riboflavin-5'-monolaurate, hereinafter referred to as "9010I";*
2. *Riboflavin-4',5'-dilaurate, hereinafter referred to as "9010II";*
3. *Riboflavin-3',4',5'-trilaurate, hereinafter referred to as "9010III"; and*
4. *Riboflavin-2',3',4',5'-tetralaurate, hereinafter referred to as "9010IV".*

The following tests are conducted for assessing the effect of various riboflavin derivatives.

**(I) *IN VITRO* HYDROLYSIS OF 9010I AND 9010IV**

**Materials**

Samples of 9010I and 9010IV: 9010I was weighed and dissolved into ethanol to prepare a suspension of 9010I in ethanol (0.7mmol/L). Similarly, a suspension of 9010IV in ethanol (0.7mmol/L) was prepared.

Supernate of liver, kidney or muscle tissue homogenate: Weight 3g of fresh liver, kidney or muscle tissue, and then add 30ml of phosphate buffer (0.2mol/L) at pH 7.4 thereto. The resultant mixture was homogenized via a homogenizer to homogenate, and centrifuged under 2100 x g for 15min.

Serum Diluent: 2ml of serum was mixed homogeneously with 8ml of phosphate buffer (0.2mol/L) at pH 7.4 to form a serum diluent.

**Methods**

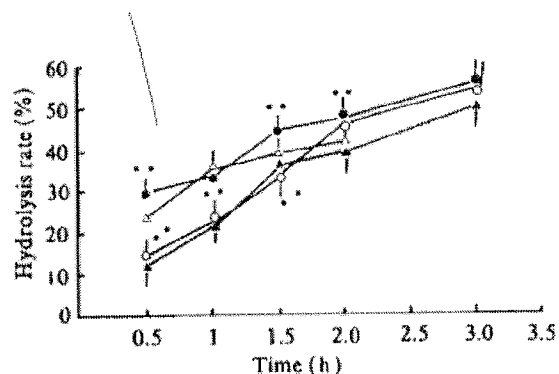
Determination of *In Vitro* Hydrolysis Rate of 9010I: 25 $\mu$ l of the suspension of 9010I in ethanol (0.7mmol/L), 0.5ml of 0.3% Tween, and 0.5ml of the supernate of liver, kidney or muscle tissue homogenate as prepared above were sequentially added to a 5ml centrifugal tube and homogeneously mixed. The aliquots of the resultant mixture stood at 37°C for 0.0, 0.5, 1.0, 1.5, 2.0, or 3.0 hours, respectively. Each aliquot was mixed with 3ml of ethyl acetate, shaken for 1min in a liquid positive mixer, centrifuged under 2100 x g for 15min, and measured for its fluorescence intensity.

Determination of *In Vitro* Hydrolysis Rate of 9010IV: 25 $\mu$ l of the suspension of 9010IV in ethanol (0.7mmol/L), 0.5ml of 0.2% Tween, and 0.5ml of the supernate of liver, kidney or muscle tissue homogenate as prepared above were sequentially added to a 5ml centrifugal tube and homogeneously mixed

to prepare a sample. 25 $\mu$ l of ethanol was used as the control. Both the sample and the control were incubated in a water bath at 37°C for 24h, and heated in a water bath at 90°C for 5 min to precipitate proteins. The sample and the control were mixed with 2ml distilled water, respectively, and centrifuged. Measure the fluorescence intensity of supernate from the sample relative to that from the control, and then calculate the content of riboflavin.

## Results

1. Hydrolysis Rate of 9010I: In any one supernate of liver, kidney or muscle tissue homogenate, the serum can hydrolyze 9010I. The hydrolysis rate of 9010I is shown in Fig. 2. It can be seen that the hydrolysis rate increases over time.



**Fig 2. The curves of hydrolysis rate of 9010 I .**

(○) liver; (●) kidney; (△) muscle; (▲) serum.

$\bar{x} \pm SD$ ,  $n=3$ . \* \*  $P<0.01$ , compared with muscle

2. Hydrolysis Rate of 9010IV: It is determined that the fluorescence peak of the sample of 9010IV has an intensity of  $26.6 \pm SD2.2$  while the fluorescence peak of the control has an intensity of  $27.4 \pm SD1.6$ . Thus, it is indicated that 9010IV can hardly be hydrolyzed *in vitro*.

## (II) Oral Administration Test for Rat

### Materials

9010I, 9010II, 9010III, and 9010IV were dissolved into the 1:1 mixture of ethyl oleate and camellia oil, respectively, to form injectable suspensions. AIN-76<sup>TM</sup> riboflavin-free feed formulation is used as the daily diets for experimental

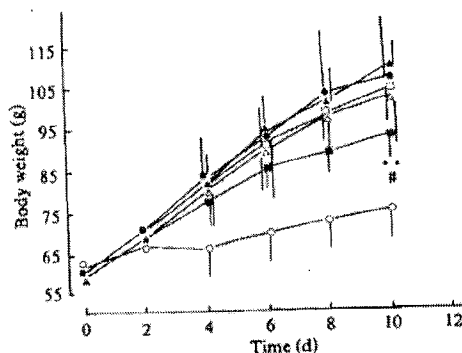
animals.

### Methods

42 Wistar rats were randomly divided into six groups, namely, riboflavin-lack group, riboflavin-control group, 9010I group, 9010II group, 9010III group, and 9010IV group. The rats in the riboflavin-lack group were orally administered with 0.5ml of mixed oil of ethyl oleate and camellia once per day, while the rest was orally administered with 0.5ml of the suspension of riboflavin, 9010I, 9010II, 9010III, or 9010IV in the mixed oil of ethyl oleate and camellia once per day, respectively. The experimental rats were fed in separate cages, and took freely the diets and water. Weigh the experimental rats every other day during a test period of 10 days.

### Results

The results were shown in Fig. 3 and Table 1. It can be seen that the rates in 9010I group have the most gain in body weight.



**Fig 3. Change of body weight.** Rats were given po 10.6  $\mu$ mol of riboflavin or 9010 and riboflavin-free diet during the experiment. (○) riboflavin-free; (●) riboflavin; (△) 9010 I; (▲) 9010 II; (□) 9010 III; (■) 9010 IV.  $\bar{x} \pm$  SD;  $n=7$ ,  $^{**}P<0.01$ , compared with riboflavin-free;  $^{\#}P<0.05$ , compared with riboflavin

**Tab 1. Diet intake in rats**

Group	n	Diet intake(g / rat)
Riboflavin free	7	70 ± 9
Riboflavin	6	110 ± 12 <sup>**</sup>
9010 I	7	110 ± 15 <sup>**</sup>
9010 II	7	113 ± 13 <sup>**</sup>
9010 III	7	111 ± 12 <sup>**</sup>
9010 IV	7	98 ± 19 <sup>**#</sup>

$\bar{x} \pm SD$ , <sup>\*\*</sup>  $P < 0.01$ , compared with riboflavin-free; <sup>#</sup>  $P < 0.05$ , compared with riboflavin

### (III) Intramuscular Injection Administration Test for Rats

#### Materials

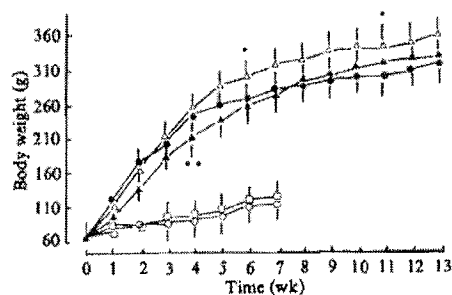
9010I, 9010II, 9010III, and 9010IV were dissolved into the 1:1 mixture of ethyl oleate and camellia oil, respectively, to form injectable suspensions. AIN-76<sup>TM</sup> riboflavin-free feed formulation is used as the daily diets for experimental animals.

#### Methods

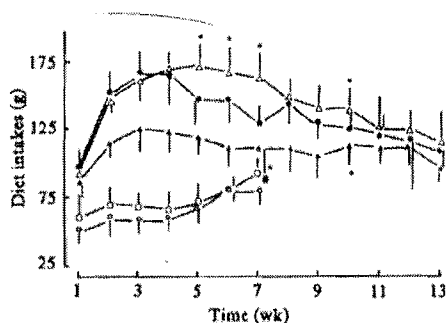
40 Wistar rats were randomly divided into six groups, namely, riboflavin-lack group, riboflavin-control group, 9010I group, 9010II group, 9010III group, and 9010IV group. The rats in the riboflavin-lack group were intramuscularly administered with 0.5ml of sterile oil mixture of ethyl oleate and camellia once per day, while the rest was intramuscularly administered with 0.5ml of the sterile oil suspension of riboflavin, 9010I, 9010II, 9010III, or 9010IV in the mixed oil of ethyl oleate and camellia once per day, respectively. The experimental rats were fed in separate cages, and took freely the diets and water. Record the diet intake of each rat, and measure their body weight every week during a test period of 3 months. Moreover, measure and record the change of activation coefficients (AC) of blood glutathione reductase (BGR) in rates.

#### Results

The results were shown in Fig. 4-5. It can be seen that 9010I has a long-acting property far better than others.



**Fig 4. Change of body weight.** Rats were given im 10.6  $\mu$ mol of riboflavin or 9010 and po riboflavin-free diet during the experiment. (○) riboflavin-free; (●) riboflavin; (△) 9010 I; (▲) 9010 III; (□) 9010 IV.  $\bar{x} \pm SD$ ;  $n=8$ , \*  $P<0.05$ , \* \*  $P<0.01$ , compared with riboflavin



**Fig 5. Diet intakes of rats.** Rats were given im 10.6  $\mu$ mol of riboflavin or 9010 and po riboflavin-free diet during the experiment. (○) riboflavin-free; (●) riboflavin; (△) 9010 I; (▲) 9010 III; (□) 9010 IV.  $\bar{x} \pm SD$ ;  $n=8$ , \*  $P<0.05$ , compared with riboflavin; #  $P<0.05$ , compared with riboflavin free

## Discussion

It can be concluded from the above-described experiments that 9010I (i.e., riboflavin-5'-laurate as claimed in the present invention) have the best long-acting nutritional effects compared with others, that may be resulted in by the combination of its specific esterification degree, esterification site and ester-forming carboxylic acid.